**THE OHIO STATE UNIVERSITY**

**William G. Lowrie Department of Chemical and Biomolecular Engineering**

**Spring 2018**

# Characterizing the Mechanical Properties of the Tumor Extracellular Matrix with a DNA Origami Sensor

**Submitted by: Kelly Kolotka**

**Presented in Fulfillment of requirements for graduating with Honors Undergraduate Research Distinction in Chemical and Biomolecular Engineering at The Ohio State University**

Thesis Committee: Dr. Jonathan Song, Advisor

Dr. Carlos Castro

Department of Mechanical and Aerospace Engineering, Comprehensive Cancer Center

## Abstract

Tumor growth and metastasis are believed to be promoted by alterations to the mechanical properties of the extracellular matrix (ECM). These mechanical alterations in tumors are also highly dynamic, but our understanding of the exact causes and consequences of tumor ECM remodeling is constrained by the current methodologies used to measure them. Therefore, the goal of this study was to better understand stability and matrix properties by developing a new sensor capable of detecting for alterations in the mechanical properties of the ECM with improved temporal and spatial resolution compared to current methodologies. To achieve this goal, we used the NanoDyn, a DNA origami nanostructure, which has been previously used to measure the effects of macromolecular crowding on the rheological properties of polyethylene glycol solutions. Our study aimed to adapt the NanoDyn for measuring stability and dynamic changes of the mechanical properties of the tumor ECM. The NanoDyn detection scheme consists of a double barrel structure which can be either in an open or closed state depending on its surroundings. The state of the device is found by fluorescence resonance energy transfer (FRET) due to the changing proximity of two fluorophores. By measuring stability and overlap concentration of well-characterized polymer solutions, the NanoDyn was initially characterized. Subsequently, stability testing for the NanoDyn in collagen and hyaluronan, which are two of the main constituents of tumor ECM, were conducted using transmission electron microscopy (TEM) and gel electrophoresis. Results from the overlap concentration measurements showed that the NanoDyn measurements coincide with accepted literature. Additionally, the NanoDyn has been found to be stable in collagen for two weeks by changing the solution composition from magnesium chloride to sodium chloride. Therefore, the NanoDyn structure has the capability of measuring biological components of the microenvironment due to early successes in stability and measurement. In future investigation, the NanoDyn in collagen and hyaluronan solutions will be analyzed using FRET to determine how these biological components impact the state of the NanoDyn. These findings would provide a better understanding of the tumor promoting properties of two of the main components of the ECM.

## Acknowledgements

First, I would like to thank Dr. Jonathan Song for his mentorship, support, and encouragement over the past two years. His never ending guidance and vision have been inspiring, and it has been an absolute honor to be a part of his research group for the past two years. I would also like to thank Dr. Carlos Castro for his instruction about the NanoDyn and for offering a very critical perspective to the project. I have deeply enjoyed getting to work on this project which is a collaborative effort between two highly achieving and understanding principal investigators.

I would also like to thank the other members on the NanoDyn team. This started with Dr. Mike Hudoba's successful creation of the device, and I appreciate the opportunity to work with it. Additionally, Sarah Bushman and Patrick Kinnunen were original members of this project team. Without their brilliant minds and great company, this project would have been overwhelming to a new researcher like myself. Morgan Hyland and Peter Beshay have been critical to the progression of this project. Since Morgan and I started at similar times, we have been able to learn from each other's mistakes, and I am grateful for all of the experiments that she contributed on as part of this thesis work. Peter's unique perspectives and positive personality have been motivational throughout the past year, and I have greatly enjoyed the opportunity to work with him.

The entire atmosphere of the Microsystems for Mechanobiology & Medicine Group as well as the Nanoengineering and Biodesign Laboratory has been a very positive experience. All of the guidance and mentorship that I have received have been critical to my growth and success.

Finally, I would like to thank my family and friends. Without their support and encouragement, I would never have had the opportunities that I have been given during my undergraduate career at The Ohio State University.

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# 1 Chapter 1: Introduction

## 1.1 Background

This thesis focuses on the development of technology for detecting and measuring the dynamic changes of the tumor microenvironment using a DNA nanotechnology sensor. In the remainder of this section, background information regarding critical aspects of this technology is presented in addition to the motivation for study in this field. Subsequently, the remaining chapters detail the experimental methods and results of testing for three separate phases. Each chapter includes more detailed background information that is critical to the content of that chapter.

## 1.2 Significance

Cancer is the second leading cause of death in the United States and accounts for half a million deaths every year [3]. One of the key challenges in treating cancer is its diversity and its rapid evolution. One of the major changes that occurs during tumor formation is the remodeling of the tumor ECM. During tumor progression, the hypoxic environment upregulates enzymes such as lysyl oxidase which increases collagen fiber formation and collagen deposition as shown in Figure 1 [1]. This leads to matrix remodeling and an increased stiffness, making a very hostile environment that is difficult to penetrate for drug delivery and other therapies. By better

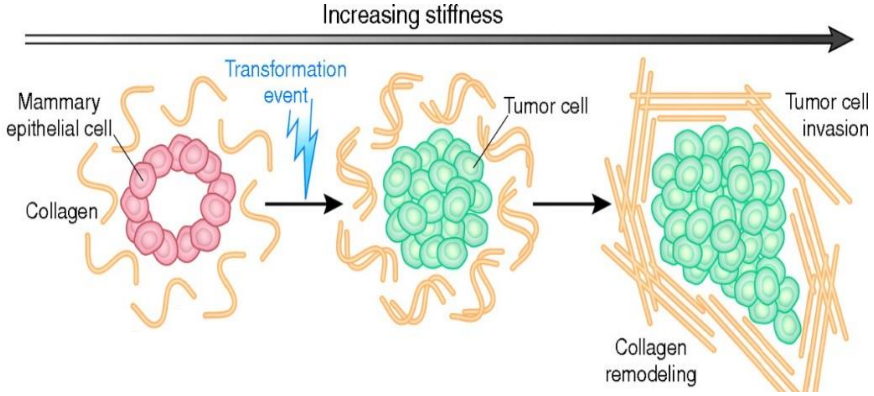


**Figure 1: Tumor environment evolution in breast cancer [1]**

understanding these changes and having engineering metrics to quantify these changes would lead to drastic improvements for both treatment methods.

Unfortunately, the current measurement techniques do not have the capability to quantify the mechanical properties of the tumor microenvironment. Either limited in scale or to basic topography, current methods include microindentation, compression testing, rheometers, optical trapping, and atomic force microscopy. For example, microindentation and atomic force microscopy can make measurements at the micro/nano scale, but they are limited in measuring surface properties. On the contrary, compression testing and rheometers can measure bulk properties of a sample, but a large quantity of sample is necessary, and the measurements are at a larger scale. Because all of these methods have their drawbacks, investigation into other technology is necessary. Nanostructures such as DNA origami have the versatility and capability of measuring diverse systems, and optimization of the NanoDyn has the potential to measure biological systems. By better understanding the stability and interactions of the NanoDyn with different solutions, progression towards dynamic measurements of the ECM can be achieved in the realm of molecular crowding, viscosity, and other rheological properties.

## 1.3 The Extracellular Matrix

The extracellular matrix (ECM) is the non-cellular component found within all tissues and organs. It is crucial for biochemical and mechanical properties in addition to providing the structural scaffold for cells and is very heterogeneous as result. As previously mentioned, the ECM drastically changes during tumor development due to changes of the components of which it is comprised. The ECM is a highly dynamic structure and its components undergo changes either enzymatically or non-enzymatically which change the viscoelastic and topographical properties [5].

The primary components of the ECM fall into two main categories: proteoglycans (PGs) and fibrous proteins. The main fibrous proteins are collagens, elastins, fibronectins, and laminins of which collagen is the primary structural component and one of the materials of focus in this research. PGs fill the majority of the ECM interstitial volume as a hydrated gel. PGs are comprised of a glycosaminoglycan, of which hyaluronan is a member, linked to specific proteins. These molecules are very hydrophilic and enable matrices about to withstand high compressive forces. They play a role in cell adhesion, migration, signaling, and proliferation. The major components of the ECM can be seen in Figure 2 [5, 12].

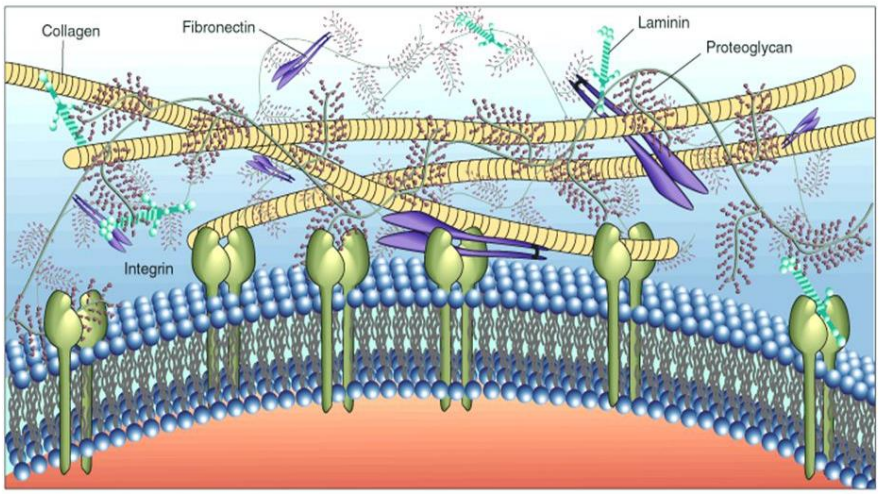


**Figure 2: Key components of the ECM. [12]**

## 1.4 DNA Origami

Deoxyribonucleic acid (DNA) molecules are biopolymers found in cells that dictate the genetic composition of cells. DNA is composed of a phosphate group, a deoxygenated ribose sugar, and a nitrogenous base. These nucleotides always have the same phosphate groups and sugar, however, the nitrogenous base can be one of four: adenine, cytosine, guanine, and

thymine. DNA is a double helical structure where the two strands are complimentary based on these base pairs. Adenine pairs with thymine, and cytosine pairs with guanine across each strand. This is due to molecular size and hydrogen bonding. The directionality is specified by a five prime (5') to three prime (3') orientation where the 5' end has a terminal phosphate, and the 3' has a terminal sugar. The 3' of one strand aligns with a complementary strand beginning at the 5' end.

The double helical structure of DNA can be found in Figure 3 [15].

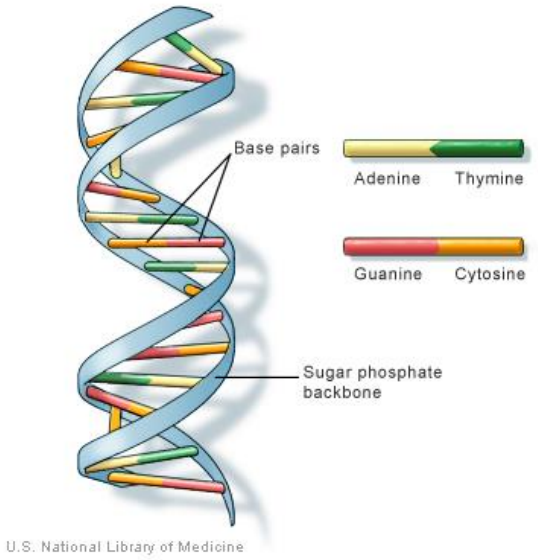


**Figure 3: Double helical structure of DNA [15]**

In 1982, Nadrian Seeman first used DNA to form nanoscale structures by manipulating Holliday junctions. These Holliday junctions were used to create repeating lattices of DNA which integrate four single-stranded DNA molecules. These molecules undergo folding reactions to reach an energetically favorable state and form 2D and 3D structures. Thus, the field of DNA origami was introduced and has since made significant progress [4, 8].

As mentioned previously, there are certain design constraints to measure the ECM that are not satisfied by current technology. Limitations in size and scale are something to consider, as the measurements need to be made at the micro/nano scale in order to maneuver and measure

certain components in the ECM. Additionally, the technology must be biocompatible since the aim is to better understand a complex biological system. In addition to size constraints, the new technology needs to be versatile to take both 2D and 3D measurements. The device also needs to be durable as the tumor environment is fairly hostile. And finally, an optimal design would be adaptable to the situation in which it is used. The team turned to DNA origami to meet these constraints as previous research indicates that it is very precise at the nanoscale, it can be modified and adapted by altering the staples used, and it is biocompatible since it uses one of the main biological molecules in the body. The main foci of this these was to investigate the plausibility of DNA origami to meet the versatility and stability requirements.

## 1.5 The NanoDyn

A previously designed DNA origami force sensor, named the NanoDyn, was developed by Dr. Mike Hudoba at The Ohio State University in the Nanoengineering and Biodesign Laboratory led by Dr. Carlos Castro. This device was the focus of all studies incorporated in this thesis, including device characterization and initial experiments with key components of the ECM. This device was chosen because it is a two state nanostructure, meaning that it can exist in an open or closed state as shown in Figure 4. The presence of two fluorophores, Cy3 and Cy5 allow for the state of the device to be detected using fluorescence [2].

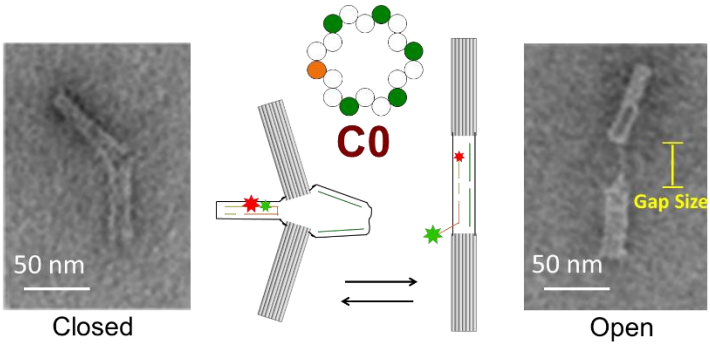


**Figure 4: Open and closed structure of the NanoDyn - schematic and TEM [2]**

The NanoDyn has a double barrel design where each barrel has a 24 helix bundle cross-section and is 50 nm in length. The two barrels are linked by six scaffold loops, and the composition of these loops can alter the dynamics of the device. Each loop is classified as a fluctuating linker, unconstrained linker, or constrained linker. The fluctuating linker contains the Cy3 and Cy5 fluorophores. When in the closed state, the device fluoresces due to the proximity of this pair. By exciting Cy3, the Cy5 emittance is measured in a system called Fluorescent Resonance Energy Transfer (FRET) which will be detailed in the next section. This fluctuating linker can be modified to control the natural state of the device. For example, by changing the DNA staple length in one of these linkers, also known as the hairpin, from 10 base pairs to 20 base pairs, a higher percent of devices would be closed when taking a static measurement [2].

The unconstrained linkers allow the device to switch between the open and closed states while constraining the linkers contain stapes that can customize certain features of the device. Certain configurations allow for flexible movement between the two states, but the addition of constraining staples can bias the device to be found in a particular state. By having the ability to alter the constraints of these five remaining linkers, the device is highly modifiable.

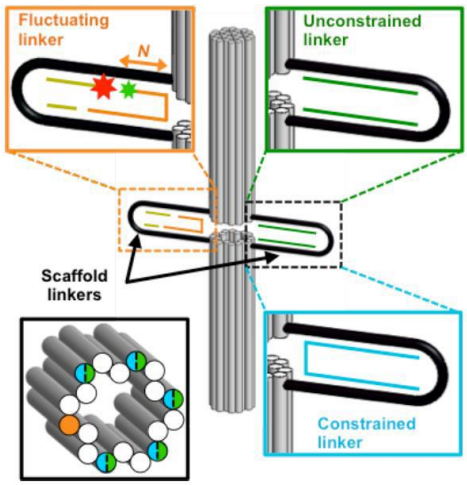


**Figure 5: Schematic of NanoDyn design. The double barrel design is connected by loops that are constrained, unconstrained, or contain a fluctuating linker [2]**

### 1.5.1 Fluorescence Resonance Energy Transfer

In order to determine the state of the NanoDyn, FRET was used. FRET is a process that is highly dependent upon distance between molecules. As previously discussed in the NanoDyn structure, there is a fluorescence-based pair of molecules on the fluctuating linker. Therefore, the dynamics of the device can be studied due to FRET efficiency as this value will increase when the two fluorescent molecules are in very close proximity, approximately 10 nm [2].

FRET uses non-radiative transfer of energy between a donor and receptor molecule. Each fluorescent molecule has an identifiable and distinguishable absorption and emission spectra. When using a spectrophotometer, wavelengths reveal the range of both excitation and emission of light for a certain fluorophore. FRET measures the overlap between the donor emission spectrum and acceptor excitation spectrum. When the two molecules are in close enough proximity, the energy from the donor can be transferred to the acceptor. The fluorophore pair for the NanoDyn uses Cy3 as the donor and Cy5 as the acceptor. These molecules have a relatively large overlap spectrum and are therefore commonly used as a FRET pair. For this reason, they were used for the fluctuating linker of the NanoDyn to indicate an open or closed structure. The following figure shows the overlap fluorescence spectra of these molecules [2].

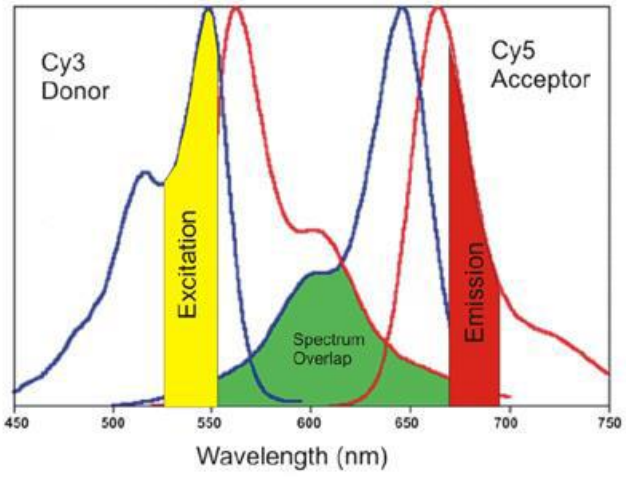


**Figure 6: FRET spectrum for the donor acceptor pair used on the NanoDyn 10]**

FRET efficiency is measured by comparing the energy transferred from donor to acceptor to the total intensity of the donor and acceptor combined. For this reason, the Cy3 and Cy5 measurements are taken for each trial. Because FRET is dependent on distance, the following formula is used to calculate the efficiency for a single FRET pair where r is the distance between the fluorescent molecules and $R_0$ is a constant based on the dye that relates the quantum yield of the donor dye, the orientation of the molecules, and the degree of overlap of the spectra of the two dyes [10] .

$$E_{FRET} = \frac{R_0^6}{R_0^6 + r^6}$$

This system is an appropriate scale to be used in the NanoDyn based on the fluctuating linker. When the fluctuating linker is bound, it is closed and results in a higher FRET efficiency compared to when it is open and the fluorophores are not in close enough proximity. Because the Cy3 and Cy5 pairings are on separate staples, one on the fluctuating linker and the other on the bound end of the scaffold loop, the FRET efficiency can be used to relate the fraction of NanoDyn in the open and close state. FRET is a useful measurement for the NanoDyn because it is compatible will biological systems, and it is easily quantifiable when taking and relating measurements.

## 1.6 Objective

The objective of the experimentation comprised in this thesis focused on three major aims: to characterize a DNA origami force sensor with known solutions, to determine the stability of the DNA origami force sensor in biological components in the ECM, and to obtain preliminary FRET measures with the sensor of biological components in the ECM.

The first aim was critical to determining the usability of the device. In order to better characterize the NanoDyn, the device was calibrated using a well-known solution. These

experiments were key to understanding what the NanoDyn could measure because the obtained data were easily compared to literature values. Moreover, the outcomes from these experiments prompted studies of molecular crowding and stability.

By achieving the second and third aims, the NanoDyn experiments became more applicable to the initial research objective which was to better understand the evolutionary nature of the ECM. In order to move toward this goal, the NanoDyn needed to be stable in these environments as well as able to take measurements. With the completion of these aims, future work can include rheological measurements with the device as well as dynamic measurements in biological environments.

# 2 Chapter 2: Molecular Crowding

## 2.1 Introduction

In order to normalize the capabilities of the NanoDyn, the structure was studied using a well-characterized macromolecule of various molecular weights. Comparisons were made for FRET efficiency and weight percent molecular crowding agent in the solution, as well as FRET efficiency and overlap concentration. The following chapter of this thesis focuses on the experiments of the NanoDyn with polyethylene glycol (PEG) to measure molecular crowding.

## 2.2 Background

According to Zhou et. al., molecular crowding refers to “effects of volume exclusion by one soluble macromolecule to another” [16]. Molecular crowding is a phenomena that occurs in highly volume-occupied environments and has the capability of altering biochemical process in that environment. Molecular crowding can impact the rates and equilibria of interactions of macromolecules which can alter the environment of the cell and the ECM [11].

When molecular crowding occurs, depletion forces arise that tend to compress the surrounding molecules or structures. Because no single species needs to be present in high concentrations, this phenomena is referred to as crowded. This is partly due to the property that macromolecules cannot interpenetrate, leading to a phenomena known as excluded volume [13]. The crowded environment has an impact on the entropy of the system due to the increased repulsions when undergoing depletion forces. When using the thermodynamic equations associated with Gibbs free energy, it takes into account the impact of changing enthalpy and entropy of the system. Enthalpy is inversely related to volume, so in order to minimize the free energy in the size, volume should be maximized when an athermal assumption is used. As shown in Figure 7, the energetically favorably condition would be for the molecules to have an

unobstructed excluded volume. However, in a crowded solution, this is not possible and an overlap volume occurs. The concentration of molecules in solution at which this occurs is known as the overlap concentration of the solution [9].

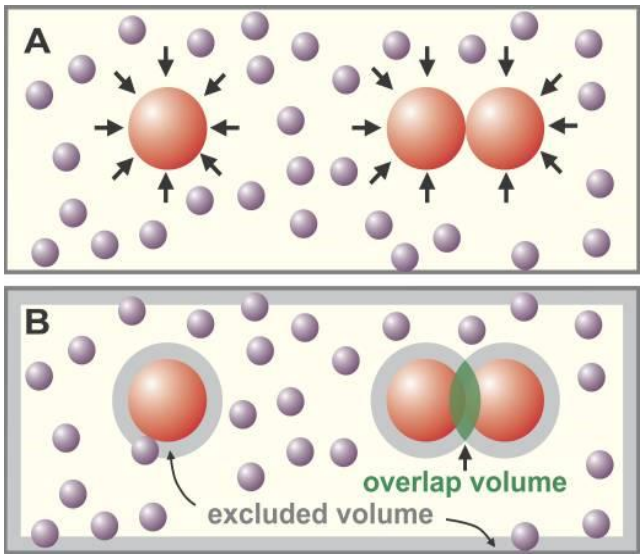


**Figure 7: Overlap volume and excluded volume in crowded environments [9]**

Overlap concentration is an important measurement for a very heterogeneous environment such as the ECM. These initial experiments with overlap concentration allowed the team to confirm that the NanoDyn sensor was capable of measuring this phenomena.

## 2.3 Polyethylene Glycol Experimental Methods

In order to better characterize the NanoDyn, experiments were done using PEG of differing molecular weights. Before this could be accomplished, the NanoDyn structures needed to be folded, purified, and verified. The NanoDyn structures were folded using a previously optimized recipe and algorithm. A 64 hour thermal ramp from 65 degrees Celsius to 4 degrees Celsius was used for the folding reaction, and an 18 mM $MgCl_2$ concentration was used to aid stability. The NanoDyn was folded with an 8,064 base long scaffold at 20 nM concentration with 10x staple excess in a folding buffer consisting of 5 mM TRIS, 1 mM EDTA, 18 mM $MgCl_2$, and 5 mM NaCl. After the structures were folded, they were verified with agarose gel electrophoresis

and transmission electron microscopy (TEM). Gel electrophoresis was run with standard NanoDyn fold, the sample (the new fold), and the 8064 scaffold. DNA is negatively charged so it moves throughout the gel when a charge is applied. The samples move based on size, so the fold could be validated by matching the band migration distance. It was also expected that the NanoDyn would migrate further than the scaffold due to being more compact. TEM images were also used to validate the fold. The TEM images allowed for photographic evidence of well-developed structures as well as the natural configuration before any solution was added.

The NanoDyn structures were then purified in order to complete experiments. This was done by adding 15% wt/v PEG-8000 to the structures along with 5 mM Tris, 1 mM ETDA, and 500 nM NaCl, centrifuging them for 25 minutes at room temperature, and then resuspending them in a solution of 1x folding buffer and 10 mM $MgCl_2$. This process removed any excess staples from the solution.

After the structures were purified, they were added to different weight percent PEG solutions in a range from 0 – 15 weight percent PEG in order to control viscosity. The NanoDyn were added at 20 nM concentration. The solution volume was balanced with a buffer solution of 1x folding buffer and 10 nM $MgCl_2$.

After the solutions were mixed, FRET was used to measure if the state of the structures were primarily open or closed. This was done using a spectrophotometer (HORIBA Fluromax fluorometer) with excitation at 510 nm and the intensity measured between 530 – 750 nm. This would allow measurement of the FRET pair. The samples were also excited at 610 to directly excite Cy5 and the intensity measured from 530-750 nm. Previously developed MATLAB code processed the data to determine the FRET efficiency while taking into account the blank spectra and the spectrum of the individual fluorophores. The entire process was repeated for 8, 20, and 35 kD PEG for molecular weight.

## 2.4 Results and Discussion

The first step in the procedure was to verify the fold to ensure that the structures were created properly. This was done using gel electrophoresis and TEM images. The gel image shown below is a verification of the structures. A stock solution of NanoDyn was compared to the newly folded batch. Due to the same distance traveled and similar intensity in the gel, this fold was considered successful. The other bands in the gel are from a ladder and the scaffold. As hypothesized, the DNA origami structures moved further than the band for the scaffold (8064) due to their more organized and condensed size.

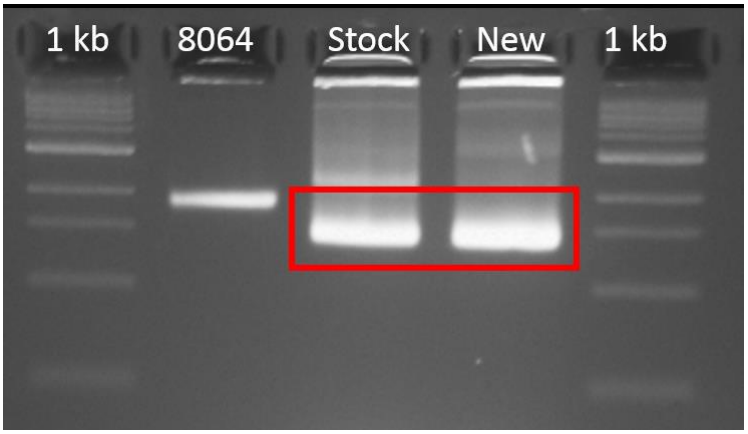


**Figure 8: Gel electrophoresis image of the ladder, scaffold, verified stock solution of NanoDyn, the new fold, and another ladder.**

Another verification method was to examine the structures using TEM images. Figure 9 shows that NanoDyn in both the open and closed configuration, indicating a successful fold.

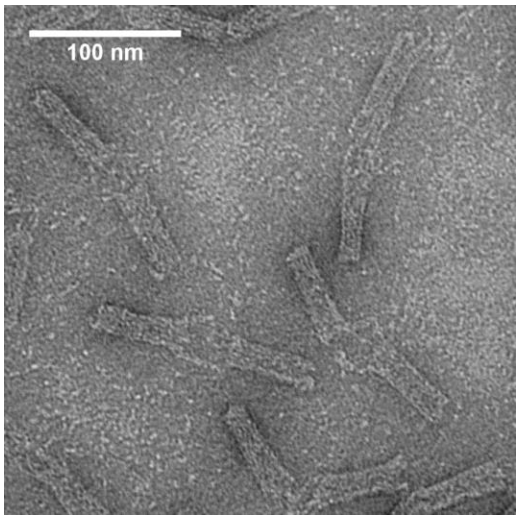


**Figure 9: NanoDyn structure using TEM**

Once the fold was verified and purified, FRET measurements were taken with the NanoDyn in different solutions of the PEG. As expected, the FRET efficiency increased when the weight percent of PEG in solution increased. When the number of molecules in solution increased, more structures were found in the closed position, corresponding to a higher FRET efficiency. This can be seen in Figure 10 and Figure 11.

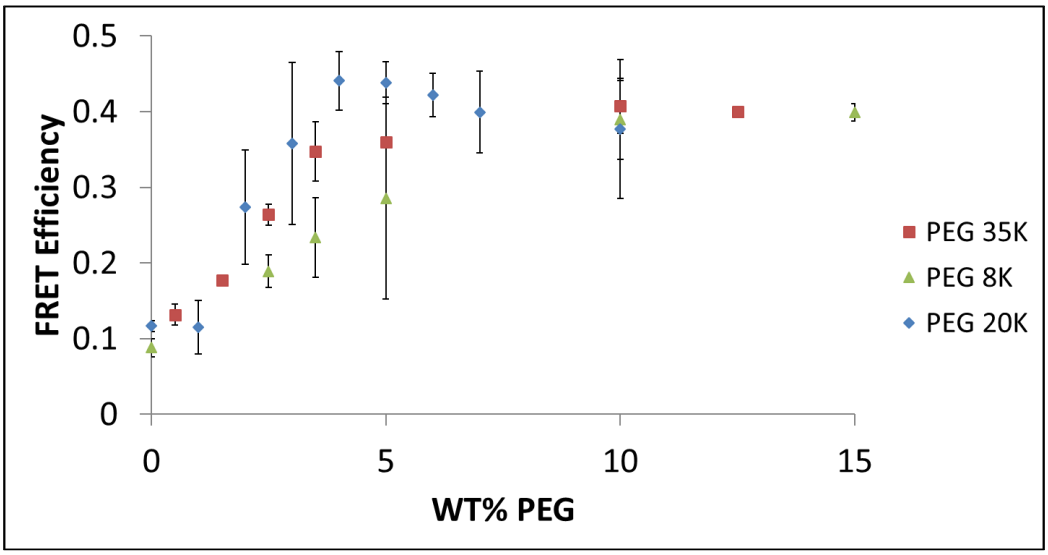


**Figure 10: Impact of weight percent PEG on FRET efficiency for three different molecular weights of PEG**

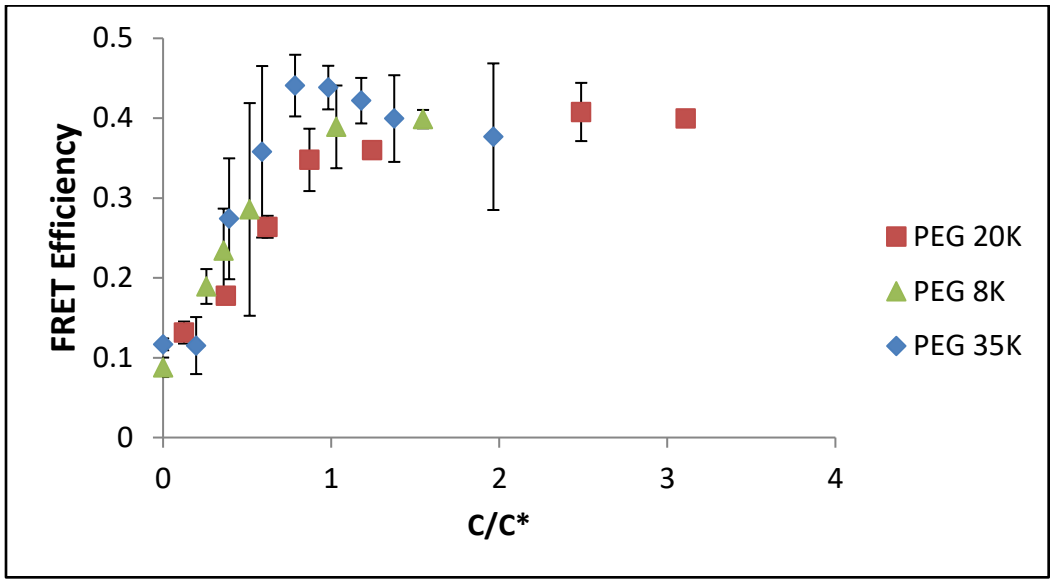


**Figure 11: Measuring overlap concentration using FRET efficiency for various molecular weights of PEG**

During this stage of experimentation, the procedure for folding, PEG purification, and verification were established. These processes were able to be applied for future systems tested. Additionally, the results suggested that the NanoDyn is, in fact, sensitive to the other molecules in solution. By following the expected trends from literature for PEG experiments, the experiments showed the potential of the NanoDyn structure. At higher viscosities of PEG, more structures were in the closed state which was as expected. The presence of additional macromolecules changed the state of the device in a way that could be measured and the expected power law relationship was observed.

Due to these studies, we determined that the NanoDyn would be capable of predicting the behavior of other solutions that would be more applicable to biological systems. The NanoDyn not only measured the expected trend, but could measure the overlap concentration. It was found that the overlap concentration of PEG 8000 is 9.7wt% from literature. This value, around 8 mPa*s, is double the point at which the NanoDyn are all closed [10]. This relationship was maintained with the other molecular weights of PEG, and could be used when studying other systems.

# 3 Chapter 3: Extracellular Matrix: Hyaluronan

## 3.1 Introduction

Hyaluronan, also commonly referred to as hyaluronic acid, is a polysaccharide found in the ECM, particularly in connective tissues. It is synthesized in the plasma membrane of fibroblasts and other cells by linking sugars to the reducing end of the polymer. Due to its roles in cell development, differentiation, and cell migration, hyaluronan is a key component when studying the ECM, particularly during tumor formation. This chapter includes studies conducted with the NanoDyn in hyaluronan solutions [7].

## 3.2 Background

As previously mentioned, hyaluronan is one of the key components of the ECM. Its production increases cell proliferation and plays a role in mitosis in addition to altering cell migration. Because all of these are hallmarks of cancer, a better understanding of hyaluronan allows for a better understanding of ECM modification during tumor formation.

Hyaluronan belongs to a family of ECM proteins known as glycosaminoglycans (GAGs). Unique to the GAG family, hyaluronans are synthesized in the inner surface of the plasma membrane. This allows for extrusion of long fibers in the ECM of $10^4$ disaccharides in length. Although the function is dependent on interactions with proteins on the presence of certain cells, it has the primary functions that were previously mentioned [6].

## 3.3 Hyaluronan Experimental Methods

The experimental methods used for hyaluronan were similar to those for the PEG experiments. After the initial folding reaction, both the stability and behavior of the NanoDyn in solution with hyaluronan needed to be verified.

Gel electrophoresis and TEM images were again used to validate the stability of the structures, however, this was done after the structures were in solution with hyaluronan rather than just the NanoDyn themselves. The hyaluronan solutions were made at 0.5 mg/mL, 1 mg/mL, and 3 mg/mL. The balance buffer had different concentrations of NaCl as well in order to determine their effect on stability. For the TEM images, a full factorial design was used to analyze the full design space of the various salt and hyaluronan concentrations.

Once the NanoDyn was confirmed as stable, FRET efficiency was used to compare the NanoDyn in the open and closed states. To determine the FRET efficiency, two different calculation methods were used. The first was a simplified equation that compared the intensity of the acceptor and donor. This can be seen in the following equation:

$$E_{rel} = \frac{I_A}{I_A + I_D}$$

Additionally, the same MATLAB code as the PEG experiments was used in order to account for the FRET emission from the Cy3 excitation, acceptor emission from Cy5 excitation, emission from Cy3 fluorophores, the acceptor and donor molar extinction coefficients, and the blanks. Since this method of analysis takes into account these other factors for normalization, it is the more accurate data processing algorithm. The following equation is the basis for this calculation method where $I_{AD}$ is the acceptor intensity following donor excitation, $I_{AA}$ is the acceptor intensity following acceptor excitation, $A_{AA}$ is the acceptor absorbance at the acceptor excitation wavelength, $A_{AD}$ and $A_{DD}$ are the acceptor and donor absorbance at the donor excitation wavelength:

$$E = \frac{I_{AD}A_{AA} - I_{AA}A_{AD}}{I_{AA}A_{DD}}$$

## 3.4 Results and Discussion

As previously mentioned, the first step of the procedure was to evaluate the stability of the NanoDyn structure in the hyaluronan solutions. This was verified using gel electrophoresis which is shown in Figure 12. The 10 base pair NanoDyn structure was used for all of the PEG experiments, so this configuration was the focus of the hyaluronan experiments. When comparing the control structure to the 10 base pair NanoDyn in hyaluronan, there was some aggregation in the gel, but the structures appeared to move about the same distance as the control. This verification was further confirmed by the use of TEM imaging which was especially necessary due to the aggregation.

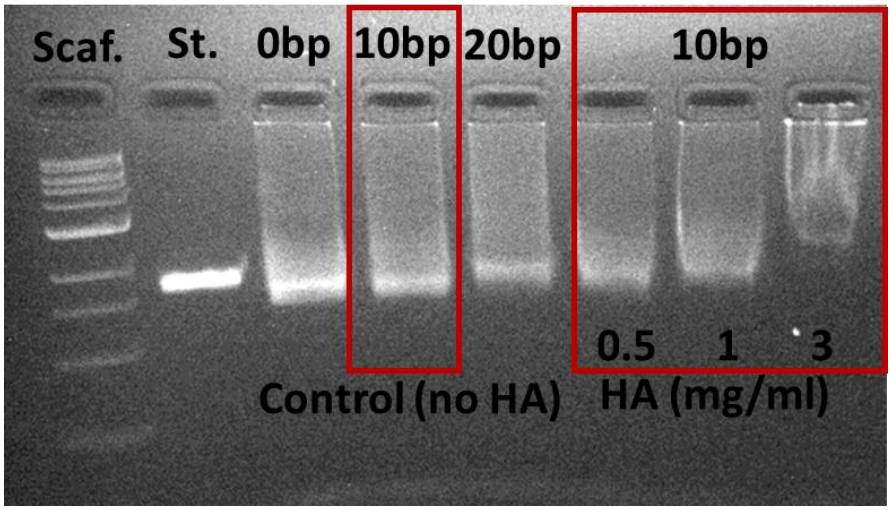


**Figure 12: Gel electrophoresis image in order to compare the stability of NanoDyn in various concentrations of PEG to the control**

In the following figure, TEM images of the NanoDyn in different solutions can be seen. The solutions varied both salt concentration and hyaluronan concentration. For all solutions, the NanoDyn were visible and stable. It was verified that the salt concentration sensitivity was not

drastically important to the stability. Because of the verified stability, the team determined that FRET analysis could then be performed.

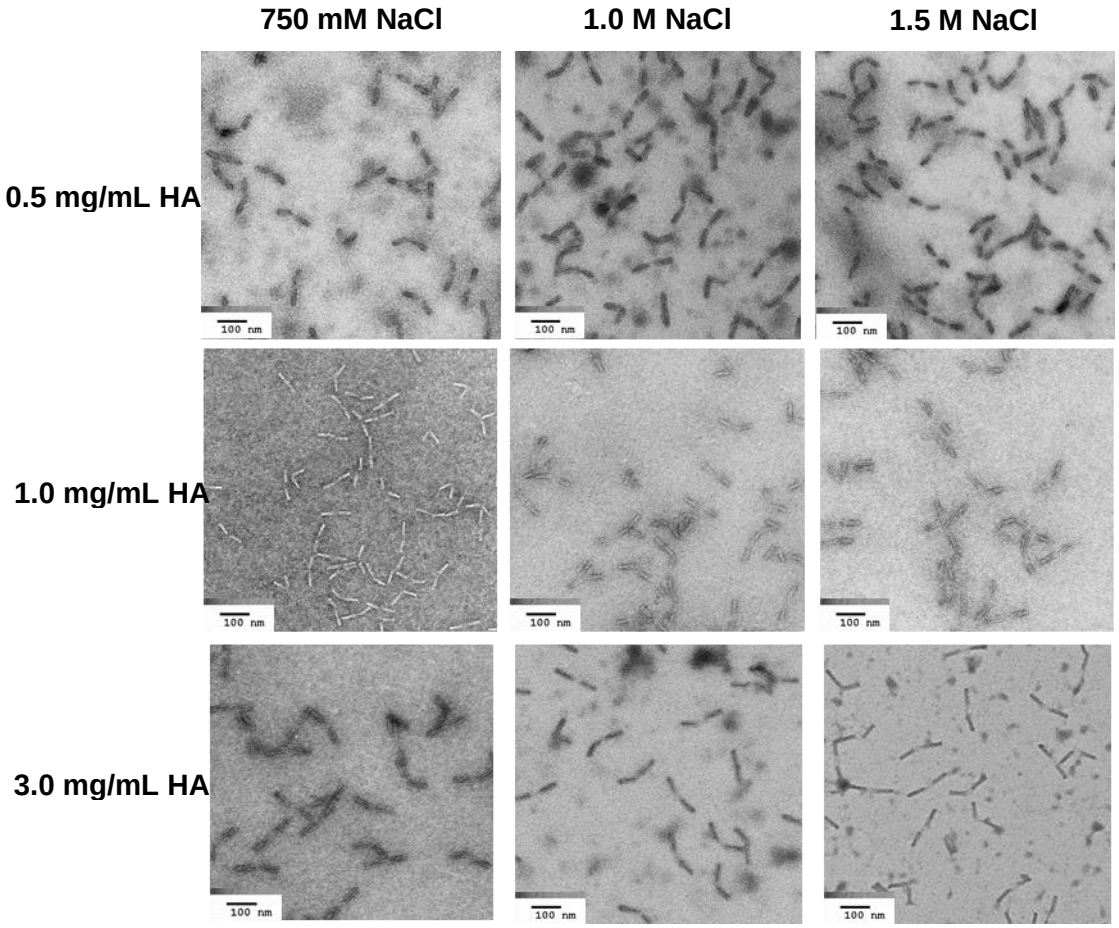


**Figure 13: TEM verification of NanoDyn structures in varying salt and HA concentrations**

By using the same spectrophotometer as the PEG experiments, FRET measurements were taken and analyzed. Figure 14 shows the spectra obtained. Figure 15 shows the FRET efficiency calculated using both the simplified method as well as the more complex calculations performed in MATLAB. Both of the calculations led to a strong linear relationship between hyaluronan concentration and FRET efficiency. This followed the hypothesis that more

hyalruonan molecules were in solution at higher concentrations, leading to increased crowding and more structures in the closed state.

These results were significant because they showed that the NanoDyn was stable in a very biologically relevant component of the ECM. Since hyaluronan is a key component of the ECM that regulates processes that impact tumor metastasis, gaining a more quantitative perspective is critical to understanding the evolution of tumor growth. By gaining these initial measurements with FRET, it opens the door for further study of viscosity and microrheology using the NanoDyn in similar solutions.

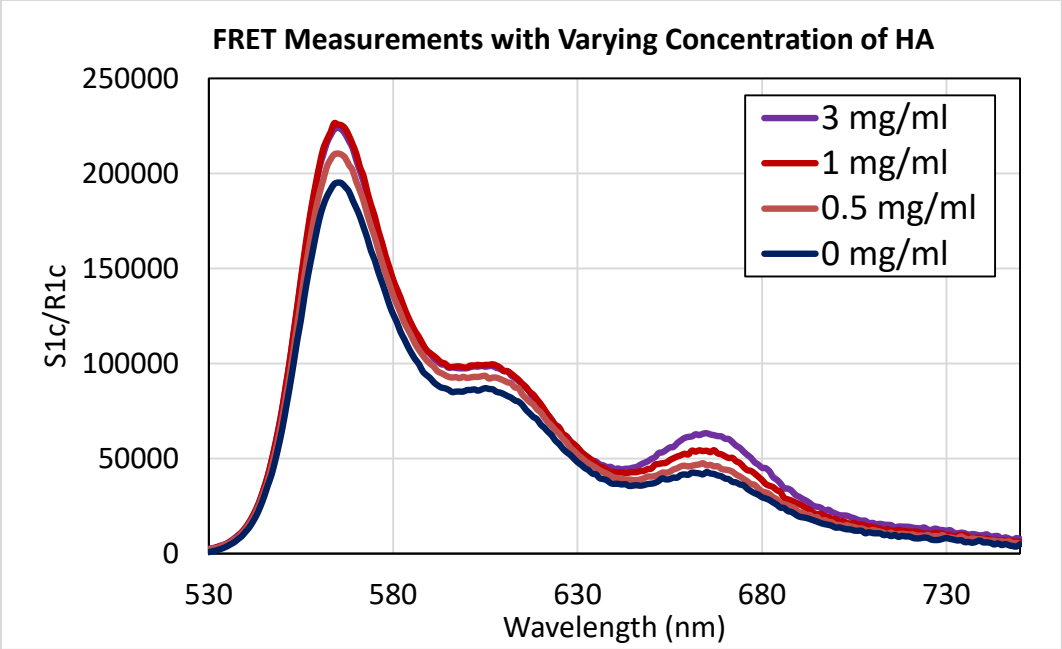


**Figure 14: FRET spectra measurements with varying concentrations of HA**

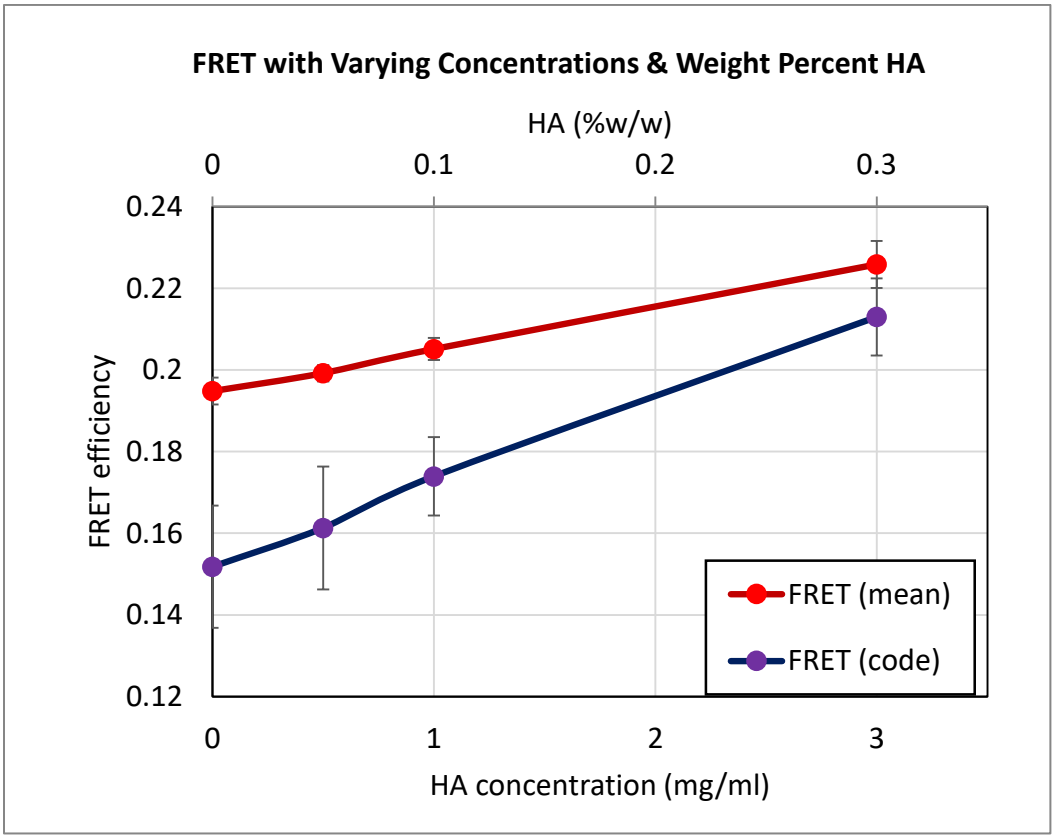


**Figure 15: FRET efficiency of the NanoDyn structure in solutions of varying HA concentration**

# 4 Chapter 4: Extracellular Matrix: Collagen

## 4.1 Introduction

Collagen is the primary structural component of the ECM and accounts for 30% of the total protein mass of multicellular animals. For this reason, collagen behavior is integral to the changes of the ECM during tumor formation. Due to its polymerization, collagen introduced new challenges in regards to methodology. The following chapter includes the attempted methodology to study the interactions of collagen and the NanoDyn as well as the successes with stability studies.

## 4.2 Background

Collagen provides tensile strength, regulates cell adhesion, supports chemotaxis and migration, and directs tissue development. There are four main types of collagen: Types I, II, and III are fibrous while Type IV is sheet-forming. The performed experiments utilized Type 1 collagen. Due to the volume of collagen present and the incorporation of the fibers, collagen is responsible for matrix remodeling [12].

During tumor formation, the microenvironment becomes hypoxic leading to an upregulation of an enzyme called lysl oxidase (LOX). LOX controls collagen cross-linking. In addition to an increase in cross-linking, there is also increased collagen deposition. The fibers become more rigid and alter the stiffness and arrangement of the ECM. These properties make collagen one of the key areas of focus for any study involving the evolution of the ECM [1].

## 4.3 Collagen Experimental Methods

Due to the complexity of collagen, many methods were attempted to incorporate the NanoDyn. They fell into two main categories: stability studies and interaction studies. As done in the previous two experiments, stability was determined first.

In order to determine the stability of the NanoDyn in collagen, similar methods of gel electrophoresis and TEM images were used. Since collagen polymerizes leading to increased viscosity, a low concentration of collagen, 1 mg/mL, was used. The procedure for making collagen can be found in literature. The NanoDyn in the standard buffer containing magnesium ions was added to the collagen solution. TEM images were then taken. However, an interesting phenomena occurred in that no NanoDyn were present in the images. This was attributed to the collagen sequestering magnesium ions which were critical for the stability of the NanoDyn. This realization led to the next group of stability studies which included changing the salt concentration and salt type and using gel electrophoresis to compare to the NanoDyn in the control buffer of magnesium. Other salts tested included 100 mM NaCl, 4 M NaCl, 500 nM KCl, and 500 nM NaCl. In addition to the gel electrophoresis, TEM images were taken.

Once the NanoDyn was stable in its new buffer solution, incorporation into collagen was attempted again. The next test of stability was to determine how long NanoDyn structures could survive in a solution of 1 mg/mL collagen. The NanoDyn in the new buffer consisting of 1x folding buffer and 100 mM NaCl was added to collagen and allowed to incubate for one month. Gel electrophoresis was run at initial introduction of sample, after 2 hours, 24 hours, 72 hours, 1 week, 2 weeks, and 1 month. The NanoDyn and collagen solution was compared to a control of just NanoDyn in the NaCl buffer using gel electrophoresis.

After the stability was determined, initial attempts of studying the interactions between collagen and NanoDyn occurred. The most promising of these methods involved centrifugation. The general collagen procedure was first followed, and the solution was allowed to polymerize. Then, the NanoDyn solution was introduced and centrifuged for 25 minutes. After this, TEM images and confocal microscopy images were taken. Flow studies were performed as well, but were unsuccessful due to an unequal distribution of NanoDyn throughout the microfluidic device.

## 4.4 Results and Discussion

When initially taking TEM images of the NanoDyn in collagen, the structures were not stable. This was attributed to the collagen sequestering magnesium ions which were necessary for structure viability. This can be seen in Figure 16.

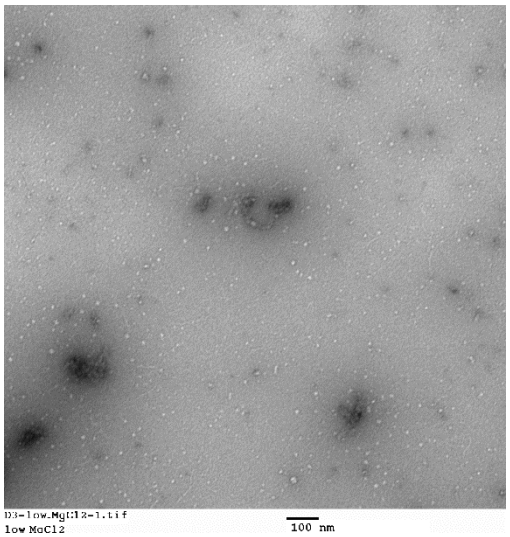


**Figure 16: TEM image of collagen and NanoDyn solution, no NanoDyn present**

To solve this problem, the composition of the NanoDyn buffer was changed. The NanoDyn in different salt buffers were run using gel electrophoresis shown in the following image. When comparing to the control of the normal buffer, the 100 mM NaCl and 4 M NaCl bands had similar distance travelled and intensity. TEM images revealed salt crystals at the higher concentrations, so a 100 mM NaCl buffer was used for all future collagen and hyaluronan testing.

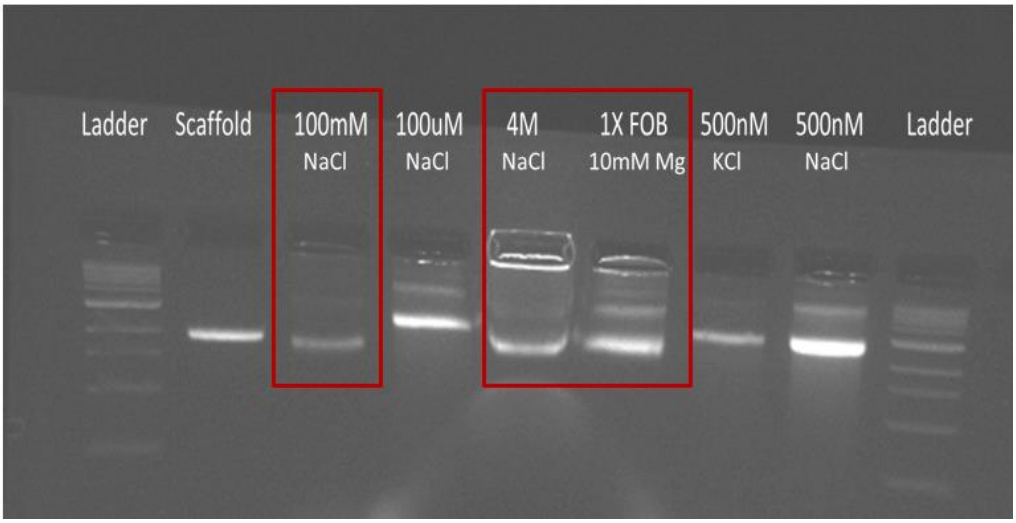


**Figure 17: Stability testing of the NanoDyn in various salt buffers**

Once the NanoDyn was deemed to be stable in the new buffer solution, it was reintroduced to collagen. Gel electrophoresis was conducted at time intervals spanning one month. All of the images taken were comparable to Figure 18. At all of the time intervals across the one month period, the NanoDyn in collagen was stable. The standard in this case was the NanoDyn in just the salt buffer solution.

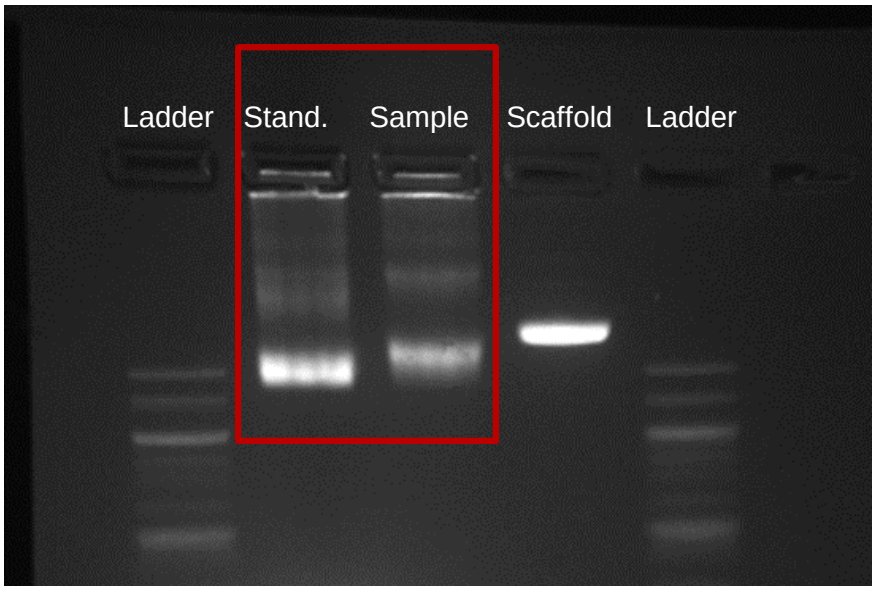


**Figure 18: Stability of NanoDyn in collagen over time**

After confirmed the stability, the goal was to study the open and closed configurations of the NanoDyn in collagen. However, due to the high viscosity of the solutions, FRET could not be used because it could not be removed from the spectrophotometer quartz cuvette once measurements were taken. Instead, the interactions were studied with TEM images. After centrifugation, a sample was extracted, crushed, and applied to the TEM grid. The following figures show viable NanoDyn in collagen using this method. The NanoDyn were found to be in

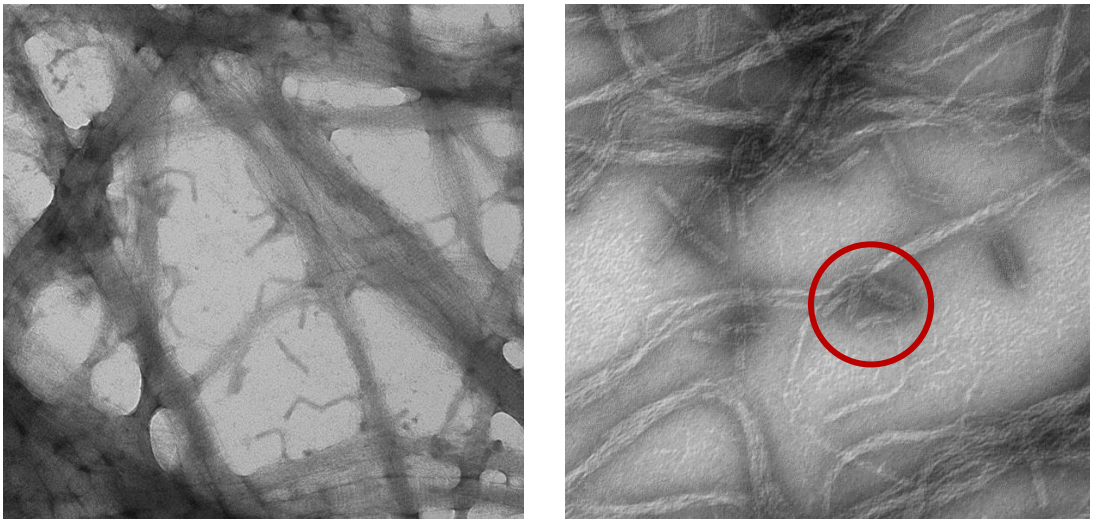

**Figure 19: Collagen and NanoDyn TEM images**

the open and closed states, and there was an attraction between the structures and the collagen fibers.

Further investigation of this centrifugation method using confocal microscopy indicated that the NanoDyn were not equally distributed in solution. Due to this heterogeneity, future investigation needs to be conducted to better study the interactions.

The collagen studies led to significant advances in the understanding the stability of the NanoDyn. Investigation led to changing the salt buffer used from magnesium chloride to sodium chloride to avoid sequestering of ions and contributing to NanoDyn stability. The success of the sodium chloride was attributed to the fact that it has a +1 charge rather than +2, but no further

investigation was conducted to confirm this hypothesis. However, this procedural modification was applied to the hyaluronan studies and will continue to be used in the future.

Additionally, the stability of NanoDyn in collagen over one month suggests that the NanoDyn is durable in biological materials. The team can now apply this knowledge in future collagen studies. Determining the efficacy of the NanoDyn device in the ECM is highly dependent on its successes in collagen. Future studies will continue to better understand this relationship by evolving the methodology.

# 5 Conclusions and Future Work

Improving our understanding of the evolution and diversity of the ECM during tumor formation may have broad-based implications for advancing cancer research. For example, we at present know surprisingly little about the oncogenic ECM remodeling events that are believed to occur during the initial stages of tumor formation. In addition, a more detailed understanding of the tumor ECM dynamics may help enhance cancer drug delivery strategies and subsequent treatment efficacy. In order to gain this understanding, new technology must be investigated due to current limitations in methods and equipment particularly in scale and 3D measurements. One proposed solution is the use of DNA origami due to its durability, compatibility, size, versatility, and capability. All experimentation in this thesis focused on the use of the NanoDyn structure due to its easily modifiable properties, two state dynamics, and quantifiable measurements using FRET efficiency.

The work encompassed in this thesis had three specific aims: to characterize the NanoDyn in a well-known solution to confirm hypothesized capabilities, to stabilize the NanoDyn in biological components of the ECM, and to obtain FRET measurements to determine if the NanoDyn could measure molecular crowding and increased viscosity. All of these aims were accomplished with similar methodology in PEG, hyaluronan, and collagen solutions.

Since collagen is such a critical aspect of the ECM and difficulties were encountered during experimentation, much of the proposed work is developing new methodologies using the NanoDyn structure in collagen solutions. The key issue of unequal distribution of NanoDyn in collagen was not recognized by TEM alone, but the confocal images proved it is an obstacle to overcome. In both flow methods and centrifugation methods, the NanoDyn was not homogenously distributed throughout the sample. This can be seen in Figure 20. The green denotes collagen fibers, and the bright yellow down the size of the right image is the NanoDyn.

Although some structures diffused throughout the collagen, the majority went down the side of the sample and congregated at the bottom. The methodology needs to be improved in order to meet the homogeneously distributed assumption made when using FRET measurements.

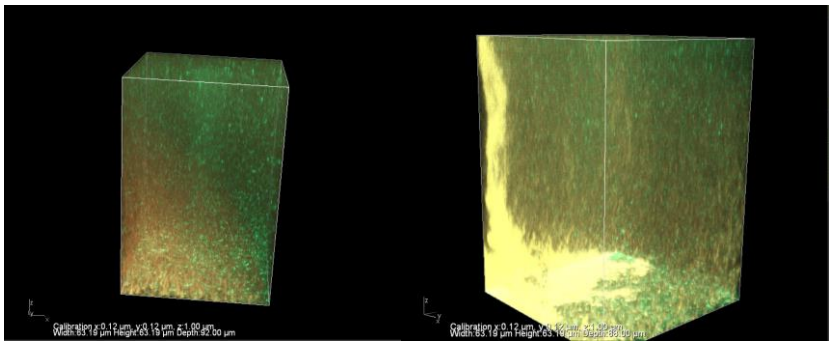

**Figure 20: Confocal microscopy images of collagen (left) and collagen + NanoDyn (right) following centrifugation method**

Once this obstacle is overcome, the NanoDyn will be ready for FRET measurements with collagen. Initial testing of disposable cuvettes to be used in the spectrophotometer show promise to aid in this effort. Collagen's polymerization does not allow for the use of the standard use quartz cuvette. In addition to static measurements, dynamic FRET measurements of both the hyaluronan and collagen solutions will be made to observe change over time.

Another interesting result of the collagen studies was that the NanoDyn were attracted and congregated to the collagen fibers. This relationship should also be studied in the future as it could be critical to understanding how the tensile forces on collagen fibers change during ECM reformation.

Finally, to further understand how the NanoDyn can measure rheological properties, particle tracking should be investigated. Figure 21 shows current techniques to measuring rheology in relation to scale and sample size. We project that the NanoDyn has the capability of measuring microrheology with a relatively small sample size. As means for comparison, particle tracking will be used as it is of similar scale and volume. Particle tracking is usually applied to

measure viscosity and elasticity of protein solutions. For this reason, it is a viable comparison for future rheological measurements by the NanoDyn.

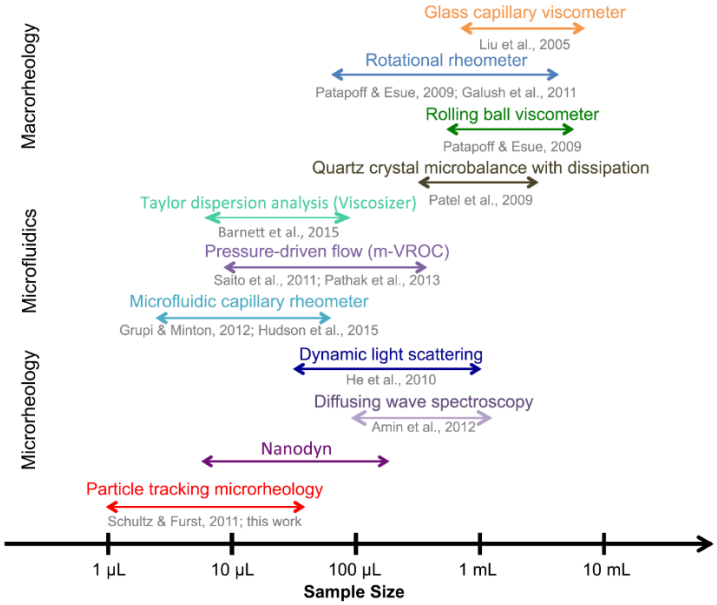


**Figure 21: Measurement techniques to measure rheology based on sample size and scale**

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